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Exposure of rotifers, crustaceans and sea urchins to produced formation waters and seawaters in the Mediterranean Sea

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The toxicity of produced formation water (PFW) originating from four natural gas production platforms located in the Adriatic Sea (Italy), and of seawater samples collected near these installations is assessed by means of bioassays with Brachionus plicatilis, *with the brine shrimp* Artemia franciscana *and with* Paracentrotus lividus. *The toxicological response of these specimens was evaluated in order to identify the most sensitive one, in the consumer compartment, and to design a bioassay battery specific for samples collected in the surroundings of a gas platform. Larval mortality of rotifers, larval immobilization of crustaceans, fertilization success/failure (sperm cell test) and larval (*pluteus*) development success/failure (embryo toxicity test) of sea urchins were taken as ecotoxicological endpoints. The PFW sampled on two platforms resulted toxic, while no toxicity was recorded in seawater samples collected in the vicinity of the platforms, even in coincidence with the PFW discharge operations. The species and bioassays employed have shown different responses to PFW:* P. lividus *turned out to be more sensitive than* A. franciscana *and* B. plicatilis. *In particular, the embryo toxicity test showed a higher toxicity than the sperm cell test.*

Keywords: natural gas platforms, produced formation water, Adriatic Sea, bioassays, toxicity assessment, rotifers, crustaceans, urchins, sub lethal endpoint, lethal endpoint

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INTRODUCTION

When it comes to evaluating the potential load of effluents or the quality of environmental matrices, the use of traditional analytical methods is not always the best choice, as many pollutants, in case of episodic input, may be active at levels difficult to be traced by ordinary techniques. The use of environmental toxicology and of ecotoxicology is becoming increasingly widespread, and may represent a fruitful integration to the physical–chemical approach. These techniques are also considered in the more recent Italian and European legislation on water protection (see, e.g. the Italian Legislative Decree No. 152 of 2006 and the European Water Framework Directive 2000/60). The traditional analytical, physical–chemical analyses provide information on the presence and quantity of pollutants. The introduction of tests on aquatic organisms yields an indication on the toxicity of matrices. The bioassays, together with the classic chemical analyses, can contribute to the definition of thresholds and limit values of pollutants in effluents and ecosystems (Damiani, 2002).

Produced formation water (PFW) is an effluent discharged from gas and oil platforms. It originates from water naturally present in geological formations (formation water) and water injected in the oil field (process water) to maintain reservoir pressure. Because of the contact with formations over geological time scales, PFW composition includes a mixture of inorganic and organic compounds (Trieff *et al.*, 1995). PFW is discharged into the sea after separation from oil and gas according to existing laws (in Italy this is ruled by the Legislative Decree No. 152 of 2006). The competent institutions monitor the PFW discharge and evaluate the possible effects on the marine ecosystem. Chemical analyses and bioassays represent a suitable tool to accurately monitor the effects of PFWs on the marine environment.

Chemical characteristics of PFWs are very unusual and the exposure of marine organisms to contaminants contained in PFW may cause different responses (e.g. narcosis, alterations of the permeability of cell membranes and developmental defects; see OGP, 2005). The integration of chemical and biological analyses allows assessment of the toxicity and bioavailability of PFWs, to understand the mechanisms of their toxic action and identification of the area of potential biological impact of PFW discharge (see review by Cianelli *et al.*, 2009).

This study evaluates the applicability of some test species belonging to the consumer compartment to investigate the toxicity of PFWs discharged from four natural gas production platforms located in the Adriatic Sea. Since the composition of PFWs over time may be altered by volatilization, adsorption and degradation (Capuzzo, 1987), the toxicity assessments were carried out by using acute or short-term chronic tests ($\leq$96 hours).

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Very few studies have been devoted to the ecotoxicological characterization of PFW originated from Italian platforms. Mariani *et al.* (2004) have published preliminary results on the response of fish exposed to PFW samples from an Adriatic platform; examining and comparing the toxic response time to unfiltered and to filtered PFW, they observed a shorter response time to the unfiltered sample, probably induced by chemical compounds associated with the solid fraction of PFW or to the ingestion and absorption of particulate matter by test organisms. Manfra *et al.* (2007) report about the effects of PFW toxicity on the marine bacterium *Vibrio fischeri* and on the sea urchin *Paracentrotus lividus*, whereas they do not detect significant effects on sediments collected near the originating platform, likely as an effect of the swift dilution process of PFW discharge.

In this paper the toxicity of PFW has been assessed using a bioassay battery composed of *Brachionus plicatilis* (Rotifera), *Artemia franciscana* (Crustacea) and *Paracentrotus lividus* (Echinodermata). The first bioassay evaluates the mortality of rotifer larvae; the second test evaluates the immobilization of crustacean larvae; the sea urchin tests assess fertilization success/failure with transmissible damage from sperm to the offspring (sperm cell test) and larval (*pluteus*) development success/failure (embryo toxicity test). We decided to use these life stages because embryos and larvae are less tolerant to pollutants than adults and therefore represent the critical life stage for toxicity tests (Martin *et al.*, 1981).

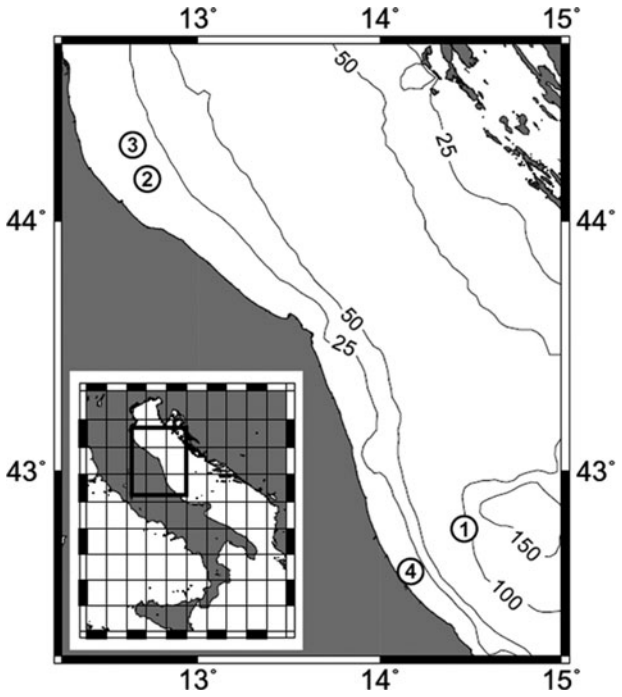

Fig. 1. Area map with sampled platforms.

MATERIALS AND METHODS

Sampling and treatment

Produced formation water samples were collected in August 2005 and September 2006 onboard four platforms located in the western Adriatic Sea (platforms PLATF1, PLATF2, PLATF3, PLATF4; see map in Figure 1). Samples, collected before discharge, were stored in high density polyethylene vessels.

Surface seawater samples were also collected, using Niskin bottles (1 l of unfiltered seawater and 1 l of seawater filtered using 0.45 μm Millipore membranes, both then stored at 4°C), in correspondence to the discharge port and 25 m downstream the observed ambient current, as suggested by Manfra *et al.* (2007): filtered samples were tested with *B. plicatilis, A. franciscana and P. lividus* while the unfiltered ones were tested only with *B. plicatilis* and *A. franciscana*. The bioassays with *P. lividus* were not carried out on unfiltered samples following the indication of Carr & Chapman (1995). For each bioassay data quality was assessed by a negative control test (with synthetic seawater for *B. plicatilis* and *A. franciscana*, and with natural filtered seawater for *P. lividus*). A positive control test (with potassium dichromate $K_2Cr_2O_7$ for *B. plicatilis*, copper sulphate $CuSO_4 \times 5H_2O$ for *A. franciscana* and copper nitrate $Cu(NO_3)_2 \times 3H_2O$ for *P. lividus*) was also carried out, in order to assess the sensitivity of species to reference toxicants (Table 1).

Bioassay methodological protocols

BRACHIONUS PLICATILIS

Rotifer cysts were hatched in synthetic seawater (with a salinity of 22 psu) and juveniles were used within 28 hours from hatching (ASTM, 1991). The nauplii were first placed in a vessel containing synthetic water with same salinity of the PFW, to enable osmotic adaptation. Then, they were transferred, with a variable-volume micropipette, to PVC multiwell plates containing the actual PFW. Each plate had 36 test wells, each of them containing 0.3 ml of PFW and five nauplii; the total number of nauplii exposed to each PFW concentration was 30. Six PFW concentrations were tested (100%, 50.2%, 25.1%, 12.6%, 6.3% and 3.1%) and each dilution was replicated six times. The mortality rate was recorded after 48 hours of incubation in the dark at 25°C. Larvae were considered dead when they did not exhibit any internal and external movement after 10 seconds. The test was considered valid only if mortality did not exceed 10% in the negative control test.

ARTEMIA FRANCISCANA

Reference cysts (RAC) were provided by the Quality Assurance Research Division US Environmental Protection Agency (Cincinnati OH 45268, USA) and by the Laboratory for Biological Research in Aquatic Pollution, University of Ghent (Belgium). Brine shrimp eggs were hatched in synthetic seawater and instar II–III larvae (nauplii) were used within 48 hours of hatching (APAT IRSA-CNR, 2003). The nauplii were transferred with a variable-volume micropipette (with a round tip, so as to allow the uptake of the larvae without damaging them) to polypropylene multiwell plates for the acute test (24 hours) and beakers for the long term acute test (96 hours). The first bioassay was carried out in a multiwell plate with 24 test wells, each of which containing 2 ml of PFW and 10 nauplii. The total number of nauplii exposed to each PFW concentration was 30. The incubation was conducted at 25°C for 24 hours and in the dark. The second bioassay was carried out in Pyrex beakers, each containing 40 ml of PFW and 10 nauplii; the total number of nauplii exposed to each PFW concentration was 30. The incubation was

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Table 1. Results of bioassays with *Brachionus plicatilis* exposed to $K_2Cr_2O_7$, *Artemia franciscana* to $CuSO_4 \times 5H_2O$ for and *Paracentrotus lividus* to $Cu(NO_3)_2 \times 3H_2O$. EC_{50} data are compared to acceptability mean value or range reported in standard methods.

Species	Reference methods	EC_{50} mean value and 95% confidence limits in negative control (mg/l)	EC_{50} acceptability mean value or range (mg/l)
B. plicatilis	ASTM, 1991	289.7 (253.5–335.5)	323 (226–420)
A. franciscana	APAT IRSA-CNR, 2003	5.9 (4.9–6.8)	<6.4
P. lividus	Volpi & Arizzi Novelli, 2001 (sperm cell test)	0.04 (0.02–0.07)	(0.039–0.071 mg/L)
	Arizzi Novelli *et al.*, 2002 (embryo toxicity test)	0.06 (0.03–0.08)	(0.051–0.087 mg/L)

conducted at 25°C for 14 hours in the light and 10 hours in the dark during the 96 hour incubation. PFW concentrations were chosen as indicated in the rotifer method and three replicates per treatment were tested. The immobilization rate was taken as endpoint. Larvae were considered immobilized if they did not exhibit any movement during observation time (15 seconds) and after mechanical stimulation. The tests were considered valid only if the control test did not exceed 10%.

PARACENTROTUS LIVIDUS

Adult sea urchins of *P. lividus* were collected by SCUBA divers in an area with low anthropogenic impact in the Gulf of Naples (Tyrrhenian Sea). Organisms were stored in a glass aquarium containing aerated natural seawater, fed on *Ulva lactuca* and *Posidonia oceanica*, at a temperature of 20°C and salinity of 38 psu, with a natural photoperiod. Spawning was induced by injection of 1 ml of 0.5 M KCl solution into the coelom through the peristome. Eggs were collected by placing spawning females separately in 250 ml beakers containing natural filtered seawater at 18°C, according to the procedures reported in His *et al.* (1999). Dry sperm from each male was collected and stored immediately in a sterile 10 ml tube placed at 4°C. Sperm mobility was checked under the microscope. For the fertilization test the protocol applied was a derivation of Dinnel *et al.* (1987), US EPA (1991) and Environment Canada (1992). For the sperm cell test, sperm was exposed to test solution (PFW and seawater samples) and incubated at 18°C for 60 minutes. Then a volume of 0.1 ml of sperm suspension was added to aliquots of 10 ml of test solution containing 1 ml of the unfertilized eggs in accordance with Volpi & Arizzi Novelli (2001). After 2 hours at 18°C, fertilization success was verified by identifying the presence of the fertilization membrane (by counting 300 eggs); after 72 hours at 18°C transmissible damage from sperm to the offspring was evaluated. The same procedure was followed to evaluate embryotoxicity; in this case the gametes were put together with a sperm:egg ratio of 10:1 according to the ASTM (1995) protocol and to Arizzi Novelli *et al.* (2002). Then 1 ml of fertilized egg suspension was transferred with a variable-volume micropipette to different polypropylene vessels containing 10 ml of test solution (six replicates per treatment). After a 72 hour period at 18°C in the dark, the percentage of *plutei* with normal (N) and abnormal development was determined by direct observation of 100 larvae per vessel, randomly chosen out of the 300 per vessel. We observed the following larval anomalies: retarded larvae with size 1/2 N (R); malformed larvae affected in skeletal or gut differentiation (P1); embryos unable to attain the *pluteus* stage as abnormal blastulae or gastrulae (P2); dead *pluteus* larvae, identified as transparent larval ghosts (D1); embryos prior to larval differentiation, e.g. pre-hatching arrest (D2). The tests were considered valid only if the percentage of fertilized eggs and normal *plutei* was $\geq$ 70%.

Data analysis

The toxicity of PFW was expressed as the percentage sample volume that induces the 50% effect (EC_{50}). The probit analysis was used to calculate the EC_{50} value with 95% confidence limits. This allowed us to analyse the relationship between a stimulus (dose) and a response (such as death or sub-lethal effects). The probit model assumes that the percentage response is related to the log dose as according to the cumulative normal distribution. The EC_{50} data were categorized according to Persoone *et al.* (1993): EC_{50} not determinable corresponding to 'non-toxic', EC_{50} values larger than 100 to 'weakly toxic', EC_{50} values between 100 and 10 to 'toxic', EC_{50} values between 10 and 1 to 'very toxic', and EC_{50} values less than 1 to 'extremely toxic'. When EC_{50} could not be calculated, we reported the maximum percentage effect normalized to the negative control.

Since the conditions of normality of data and homogeneity of variance have not been met, a non-parametric analysis (Spearman test) was used. It was applied to the data of percentage effect observed in undiluted PFWs in order to assess: (a) if there were significant differences among the five bioassays (*B. plicatilis* test, *A. franciscana* 24 hour test, *A. franciscana* 96 hour test, *P. lividus* fertilization test and *P. lividus* embriotoxicity test) applied to filtered PFWs; (b) if there were significant differences among the three bioassays (*B. plicatilis* test, *A. franciscana* 24 hour test and *A. franciscana* 96 hour test) applied to unfiltered PFWs; and (c) if there were significant differences between the PFWs that showed toxic effects.

RESULTS

In the first (August 2005) and second survey (September 2006), negative control tests using dilution water showed percentage effects less than 10% in the bioassays with rotifers and crustaceans and less than 30% in the tests with urchins, as required by methodological protocols. Copper and chromium, used as reference toxicants (positive controls), showed data within the EC_{50} acceptability range reported in the standard methods (Table 1).

Produced formation water samples collected at PLATF1 and PLATF4 did not show toxicity (maximum percentage effect was $\leq$ 10%, i.e. the organism response was comparable to that observed in negative controls) while samples from PLATF2 and PLATF3 showed toxic response only in some cases (Table 2). In particular, the *Artemia* 24 hour test and the rotifer 48 hour test never highlighted toxicity of filtered PFW (maximum percentage effect resulted $\leq$ 10%) while

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Table 2. Results of bioassays expressed as EC_{50} with confidence limits or, if it is not calculable, as mortality percentage (%M) and immobilization percentage (%I).

Species	Bioassay	Exposure	Filtered PFW				Unfiltered PFW			
			PLATF1	PLATF2	PLATF3	PLATF4	PLATF1	PLATF2	PLATF3	PLATF4
Brachionus plicatilis	Mortality test	48h	%M < 10	%M < 10	%M < 10	%M < 10	%M < 10	%M < 10	EC50 = 28.3% (23.5–34.2)	%M < 10
Artemia franciscana	Immobilization test	24h	%I < 10	%I < 10	%I < 10	%I < 10	%I < 10	%I = 30	%I < 10	%I < 10
A. franciscana	Immobilization test	96h	%I < 10	%I = 14	%I < 10	%I < 10	%I < 10	EC50 = 26% (22.3–28.7)	%I = 37	%I < 10
Paracentrotus lividus	Sperm cell test	2h	NA	EC50 = 30.3% (19.9–44.0)	EC50 = 100% (86–140)	NA	NA	NA	NA	NA
P. lividus	Embryo toxicity test	72h	NA	EC50 = 4.3% (4.2–4.4)	EC50 = 6.5% (6.2–6.9)	NA	NA	NA	NA	NA

NA, not analysed.

the *Artemia* 96 hour test showed low toxicity of filtered PLATF2 (maximum percentage effect = 14%). Sea urchin tests pointed out toxic (sperm cell test) and very toxic (embryo toxicity test) effects of PLATF2 and PLATF3 samples (EC_{50} values of 30.3% and 100% for the sperm cell test and of 4.3% and 6.5% for the embryo toxicity test). In particular, *P. lividus* showed larval malformations (P1 and P2) at 25.1% concentration of PFW and total embryo mortality (D1 and D2) with the undiluted sample (Figures 2 & 3).

The non-parametric analysis was applied only to the PFWs that showed toxicity, i.e. to samples from PLATF2 and PLATF3. The results of the filtered PFWs highlighted significant differences between the five bioassays but did not record significant differences among the two PFWs. The results relative to the unfiltered PFWs did not show significant differences among the three bioassays used nor between the two PFWs. The *Artemia* 24 hour test showed weak toxicity only of the unfiltered PLATF2 sample (maximum percentage effect of 30%), while the 96 hour test showed toxicity of the PLATF2 sample and low toxicity of the PLATF3 one (maximum percentage effect of 37%). The rotifer test (48 hours) highlighted toxicity of the unfiltered PLATF3 sample.

During the discharge of PFW, no toxic effect was recorded on seawater; the percentage effects were ≤ 10% both for seawater sampled in correspondence to the discharge and for samples collected 25 m from it. In particular, no toxicity was observed at sea after discharge for the toxic PFWs (samples from PLATF2 and PLATF3).

DISCUSSION AND CONCLUDING REMARKS

Since the chemical composition of PFW is complex and variable (Manfra *et al.*, 2007), it is very difficult to discriminate and quantify the toxicity or biological and ecological impact of one contaminant type as opposed to another (Higashi *et al.*, 1992). Consequently, we chose to study the toxicity of the whole PFW rather than of its individual chemical constituents.

Many authors (Somerville *et al.*, 1987; Brendehaugh *et al.*, 1992; Krause *et al.*, 1992; Krause, 1993; Schiff *et al.*, 1992; Stagg *et al.* 1995; Stromgren *et al.*, 1995; Mariani *et al.*, 2004) studied the toxicity of PFW *in toto* with organisms of different taxonomic groups, such as *Photobacterium phosphoreum* (Beijerinck, 1889), Lehmann & Neumann 1896, *Skeletonema costatum* (Greville) P.T. Cleve, 1878, *Americamysis bahia* Molenock, 1969, *Mytilus edulis* Linnaeus, 1758, *Crassostrea gigas* (Thunberg, 1793), *Salmo gairdneri* Richardson, 1836, *Acartia (Acanthacartia) tonsa* Dana, 1849, *Tisbe battagliai* Volkmann-Rocco, 1972, *Strongylocentrotus purpuratus* (Stimpson, 1857) *Neanthes arenaceodentata* (Moore, 1903) and *Dicentrarchus labrax* (Linnaeus, 1758). They observed EC_{50} values ranging between 4.0 and 53.5%; the Californian sea urchin *Strongylocentrotus purpuratus* was the most sensitive organism whereas the polychaete *Neanthes arenaceodentata* was the most tolerant one.

In this study, we observed EC_{50} values ranging between 4.3% and more than 100%. In particular, we found that the sea urchin (*P. lividus)* was the most sensitive organism, whereas the brine shrimp (*A. franciscana)*, after 24 hours' exposure was the most tolerant one. Moreover, the embryo

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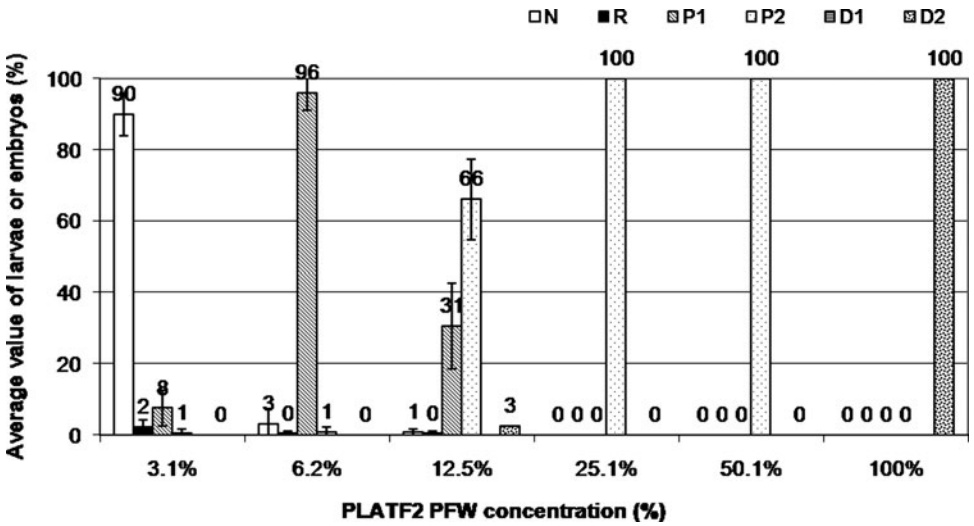

Fig. 2. Average value of normal (N) and abnormal *plutei*/embryos (retarded R, skeletal or gut malformations P1, blastulae or gastrulae P2, dead *pluteus* larvae D1, pre-hatching arrest D2) of *Paracentrotus lividus* exposed to filtered PFW collected at PLATF2.

toxicity test with *P. lividus* showed higher toxicity than the sperm cell test with sea urchins, as reported by other authors (Arizzi Novelli *et al.*, 2003; Volpi *et al.*, 2005). Therefore, PFW resulted toxic for sea urchin in terms of both embryogenesis and fertilization success although offspring quality was not affected by the exposure of sperm to PFWs. These results are in agreement with those by Schiff *et al.* (1992), Krause *et al.* (1992) and Krause (1993). In particular Krause *et al.* (1992) and Krause (1993) hypothesized that PFW contaminants could cause lesser sperm motility while the adult sea urchin females accumulate these contaminants in the eggs. This detoxification mechanism explains the toxic effects observed in adults but associated with a higher toxicity during fertilization. A similar result was also observed in molluscs (Fan *et al.*, 1992), amphipods (Linden, 1976), fish (Larsson *et al.*, 1993; Hogan & Brauhn, 1975; Daniels & Means, 1989), birds (Ratcliffe, 1967) and mammals (Britt & Howard, 1983).

The bioassays with *A. franciscana* and *B. plicatilis* highlight higher toxicity of unfiltered PFWs than of filtered samples. Mariani *et al.* (2004) observed an analogous result with fish larvae (*D. labrax*) and they attributed it to particle mechanical effects (i.e. absorption through body surface and gills or oral ingestion/digestion) and/or to particle-associated contaminants.

Usually, *Artemia franciscana* and *Brachionus plicatilis* are suitable test species because they do not require a continuous maintenance of stock cultures. On the other hand, for PFW toxicity assessment, the bioassays with *B. plicatilis* (48 hours) and *A. franciscana* (24 hours) were not particularly sensitive, and thus not very suitable to highlight the toxicity of filtered PFW. On the contrary, the *A. franciscana* 96 hour test revealed toxicity of both filtered and unfiltered PFWs.

Sea urchin bioassays utilizing sperm, eggs and fertilization reaction of echinoids require organism collection and laboratory maintenance but are fast, sensitive and inexpensive; they are used to assess the toxicity of metals, pesticides, petroleum

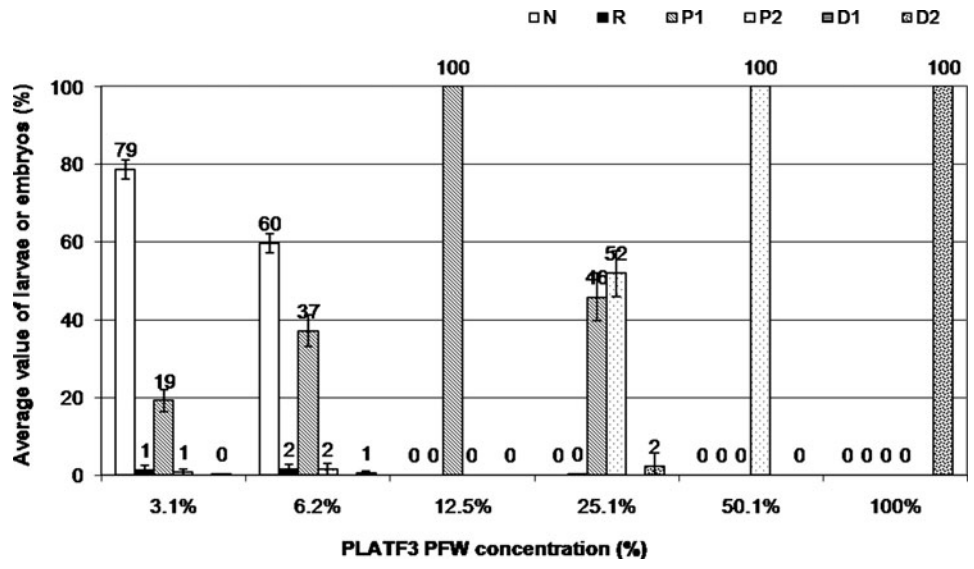

Fig. 3. Average value of normal (N) and abnormal *plutei*/embryos (retarded R, skeletal or gut malformations P1, blastulae or gastrulae P2, dead *pluteus* larvae D1, pre-hatching arrest D2) of *Paracentrotus lividus* exposed to filtered PFW collected at PLATF3.

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fractions and dispersants, sewage effluents and natural seawater. In the case of PFW, *P. lividus* was the most sensitive organism.

As an overall toxicity response, the unfiltered PLATF2 and PLATF3 samples resulted toxic with *A. franciscana* (96 hours) and *B. plicatilis*, respectively; moreover these PFWs, even if filtered, resulted very toxic from the embriotoxicity test with *P. lividus*.

What can explain the observed toxicity? The chemical composition of the PFWs has been analysed preliminarily and is reported in Manfra (2007): looking in particular at metals, volatile organic hydrocarbons (BTEX) and at the additive diethylene glycol (DEG). The results showed indeed a high content of zinc and no negligible concentrations of BTEX and DEG both in the PLATF2 and PLATF3 samples; in particular, zinc is the primary element in PFW particulate and is also associated with maintenance activities (e.g. involving the use of galvanic anodes) (Manfra *et al.*, 2007). These compounds could originate PFW toxicity probably in synergy with other contaminants present in the PFWs. In addition to the toxicity detected in unfiltered PFW and linked to particulate matter compounds, toxicity of filtered PFWs has been also recorded, indicating that some contaminants present in the dissolved phase could also generate toxic effects.

As to PFWs, *Artemia* (long-term acute test) can be used as a screening test species to evaluate the toxicity of filtered and unfiltered PFWs: if the goal is to register toxicity, it would be superfluous to use any other organism belonging to the consumer compartment; on the other hand if *Artemia* did not register toxicity, it would be obviously essential to use *P. lividus*.

Finally, as far as seawater samples are concerned, they never resulted in being toxic, independently from the quantity of discharged PFW and from the diffuser depth. As a matter of fact, different conditions of discharge showed the same behaviour of PFWs at sea: a low volume and a superficial level of discharge for the PLATF4, a high volume and a superficial discharge for the PLATF1, medium/high volumes and deep discharges for the PLATF2 and PLATF3 did not induce toxicity in the seawater receiving the discharges. No toxicity of seawater is probably a consequence of a fast and efficient initial dilution of PFW into the sea (Cianelli *et al.*, 2008) and the discharge conditions are likely to influence the dilution process rather than seawater toxicity. This is confirmed by the fact that no toxicity was observed on sediments collected near to the platform discharges, as reported in a previous study by Manfra *et al.* (2007).

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